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Effect of k_La on the Production of Glucose 6-Phosphate Dehydrogenase from *Saccharomyces cerevisiae* Grown by Fermentation Process

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Abstract

In a 5-L fermentor (NBS-MF 105), *Saccharomyces cerevisiae* (0.7 g/L) was inoculated into a liquid medium (pH 4.0) containing 17 g/L of glucose, 2.55 g/L of yeast extract, 4.25 g/L of peptone, 2.04 g/L of $Na_2HPO_4{\cdot}12H_2O$, 4.34 g/L of $(NH_4)_2SO_4$ and 0.064 g/L of $MgSO_4{\cdot}7H_2O$ and aerobically cultivated at 35°C for 22 h. Agitation and aeration were adjusted to attain initial k_La values of 15, 60, 135, and 230 h^{-1}. The glucose 6-phosphate dehydrogenase (G6PDH) productivity (Pr_{G6PDH}) obtained for *kLa* values of 15, 60, 135, and 230 h^{-1} was 10.6, 31.8, 30.3, and 23.3 U/([L·h]), respectively, whereas the cell productivity (Pr_X) for the same k_La values were 0.24, 0.69, 0.69, and 0.49 g/[L·h], respectively. Thus, both events are coupled and depend on the dissolved oxygen in the medium.

Index Entries: Glucose 6-phosphate dehydrogenase; *Saccharomyces cerevisiae*; fermentation; volumetric coefficient of oxygen transfer.

Introduction

Glucose 6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) is the first enzyme of the pentose phosphate pathway, which catalyzes the oxidation of glucose 6-phosphate (G6P) into 6-phosphogluconate. It is widely distributed in nature, being found in almost all animal tissues and micro-

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organisms. This enzyme presents great interest as an analytical reagent since it is used in many quantitative assays, including the measurement of hexokinase and creatin-kinase activities, adenosine triphospate (ATP) and hexose concentration, as well as marker for enzyme immunoassays *(1)*. The use of G6PDH for measuring glucose in the presence of fructose constitutes an important tool for the detection of illegal addition of sugar in the final products of the wine and fruit juice industries *(2)*. However, industrial production of (G6PDH) can only be viable if this enzyme is available in large quantities at competitive market prices. Although G6PDH can be attained from several microbial species, in Brazil, the use of *Saccharomyces cerevisiae* as source of this enzyme and other products *(3)* is economical sense, owing to significant experience in handling this strain in industrial ethanol plants. In addition, coupling the yeast processing with ethanol production should potentially have a positive effect on the profits of distilleries.

Because G6PDH is a constitutive enzyme, its production from a microorganism is linked to the amount of biomass obtained from a fermentation process. However, attaining a high amount of cell mass depends on the pH, temperature, constituents of the culture medium, and availability of oxygen to the cells *(4)*. For a facultative microorganism such as *S. cerevisiae*, oxygen has a crucial role in its overall metabolism, because it participates in the generation of energy through the respiratory chain inside the mitochondria. Therefore, the aim of the present study was to verify the influence of oxygen transfer rate from the gaseous to the liquid phase, expressed as k_La (volumetric coefficient of oxygen transfer), on the production of G6PDH by *S. cerevisiae*.

Materials and Methods

Chemicals

Hexokinase, ATP, nicotinamide adenine dinucleotide phosphate (NADP), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, G6P, glucose, and sucrose were obtained from Sigma (St. Louis, MO). Yeast extract and peptone were obtained from Difco (Detroit, MI). All other chemicals were of analytical grade.

Preparation of Inoculum

S. cerevisiae (isolated from pressed yeast cake) was maintained at 4°C on agar slants containing 23.0 g/L of nutrient-agar (Difco) and 1.0 g/L of glucose. The cells were transferred to 250-mL Erlenmeyer flasks containing 50 mL of autoclaved (121°C for 15 min) growth medium (GM), and the culture was incubated in a shaker (New Brunswick, Edison, NJ) for 11 h at 35°C, at an initial pH of 4.5 (adjusted with 0.5 M H_2SO_4 or 0.1 M NaOH) and agitation of 175 rpm. The composition of GM was : 3.8 g/L of glucose, 28.5 g/L of sucrose, 3.0 g/L of yeast extract, 5.0 g/L of peptone, 2.4 g/L of $Na_2HPO_4{\cdot}12H_2O$, 0.075 g/L of $MgSO_4{\cdot}7H_2O$, and 5.1 g/L of $(NH_4)_2SO_4$.

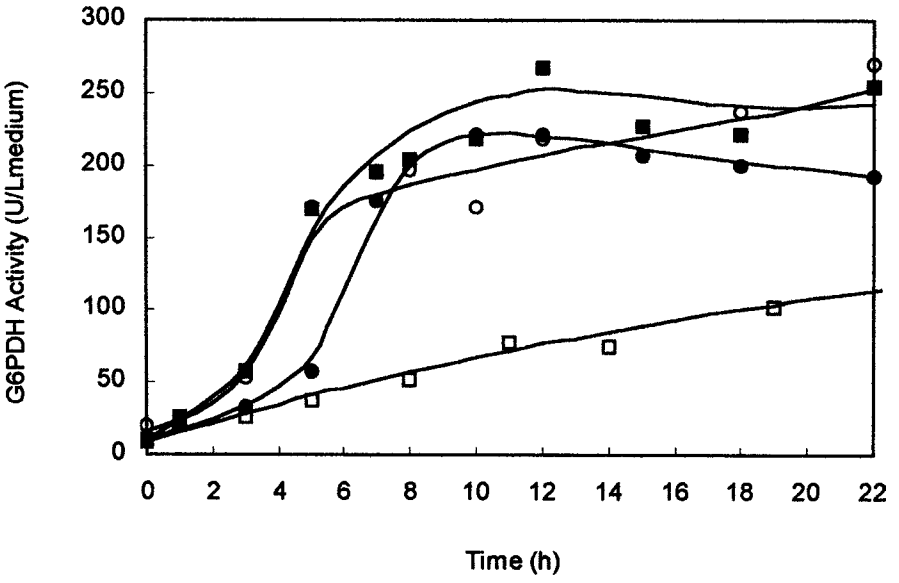

Fig. 1. G6PDH production against time for *S. cerevisiae* grown at different k_La (h^{-1}) : 15 (□), 60 (■), 135 (○) and 230 (●).

Batch Fermentation

A volume of 0.45 L of inoculum (about 4.7 g of dry mass/L) was transferred to a 5-L fermentor (NBS-MF 105, coupled with NBS dissolved oxygen controller DO-81; New Brunswick) containing 2.55 L of the culture medium (3.0 g/L of yeast extract, 5.0 g/L of peptone, 20.0 g/L of glucose, 2.4 g/L of $Na_2HPO_4{\cdot}12H_2O$, 0.075 g/L of $MgSO_4{\cdot}7H_2O$ and 5.1 g/L of $[NH_4]_2SO_4$). Fermentation was carried out at 35°C and pH 4.0 (controlled automatically by adding 0.5 M H_2SO_4 or 0.1 M NaOH) with agitation and aeration automatically adjusted to attain an initial k_La of 15 h^{-1} (0.7 vvm, 200 rpm), 60 h^{-1} (1.7 vvm, 400 rpm), 135 h^{-1} (2.3 vvm, 600 rpm) and 230 h^{-1} (2.3 vvm,800 rpm). The agitation:aeration ratio related to a desired k_La was fixed against distilled water and evaluated by using a dissolved oxygen controller (NBS-DO81; New Brunswick) in conjunction with a galvanic dissolved oxygen probe (Mettler-Toledo, São Paulo, SP, Brazil). The initial k_La was calculated using the conventional Pirt's mathematical model, cited by Wise *(5)*. To control the foam, a 10% (w/w) aqueous silicone emulsion (FG-10; Dow Corning, NY) was added dropwise. Samples were collected periodically to monitor the changes in cell density, glucose and ethanol concentrations, as well as G6PDH activity (after cell disruption). All fermentation assays were always made in duplicate, so that each solid circle in Figs. 1–4 resulted from the average of two values. The accepted difference between the experimental values was not >8%.

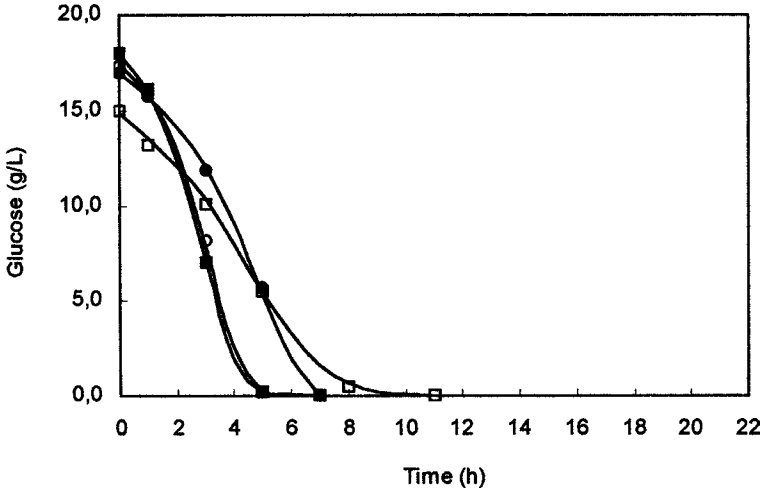

Fig. 2. Variation in glucose concentration against time for *S. cerevisiae* grown at different k_La (h^{-1}): 15 (□), 60 (■), 135 (○) and 230 (●).

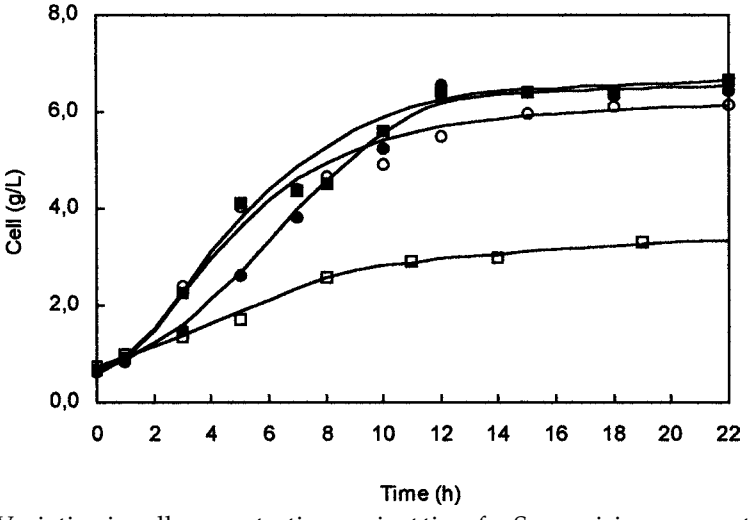

Fig. 3. Variation in cell concentration against time for S.cerevisiae grown at different k_La (h^{-1}): 15 (□), 60 (■), 135 (○) and 230 (●).

Measurement of Cell, Glucose, and Ethanol Concentrations

One milliliter of fermenting medium was centrifuged (8720*g* for 10 min) (Jouan centrifuge BR4i; Saint Herblain/France) and the supernatant discarded. The cell cake was suspended in 50 mL of distilled water and the turbidity measured with a spectrophotometer at 600 nm (Perkin Elmer-552; Perkin Elmer, Bethesda, MD). The cell concentration was estimated by comparing the optical density (OD) of the cell suspension against a standard curve for OD vs dry cell mass.

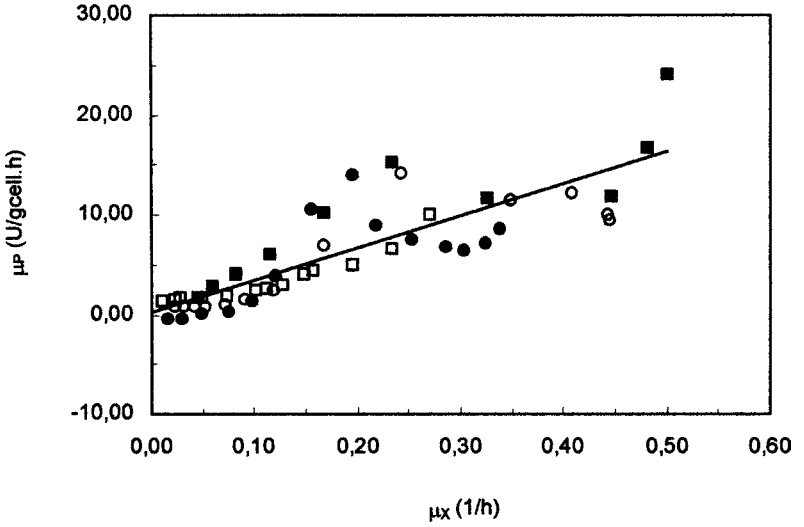

Fig. 4. Variation in G6PDH specific production rate (μP) against specific growth rate (μ_X) for *S. cerevisiae* grown at different k_La (h^{-1}): 15 (□), 60 (■), 135 (○) and 230 (●).

Ten milliliters of fermenting medium was centrifuged (2880*g* for 10 min), and the cell cake was stored at 4°C until disruption. The glucose and ethanol concentrations in the supernatant were measured by the GOD/POD enzymatic kit (Kit no.02200; Laborlab, Guarulhos, São Paulo, SP, Brazil;) and reducing dichromate back-titration method *(6)*, respectively. Each determination was made in triplicate and the standard deviation (SD) was 2%.

Measurement of G6PDH Activity

The cell cake was suspended in 50 m*M* Tris-HCl buffer (pH 7.5) containing 5 m*M* MgCl2, 10 m*M* β-mercaptoethanol, 2 m*M* aminocaproic acid, 1 m*M* PMSF, and 0.2 m*M* EDTA. The cell suspension was disrupted by vortex mixing (Phoenix AP 56; São Paulo, SP, Brazil) in the presence of glass beads (diameter = 0.5 m*M*) at low temperature (<10°C). The wet cell cake, glass beads, and Tris-HCl buffer were mixed in a volumetric proportion of 1:1:1. Cell debris and glass beads were removed by centrifugation (2880*g* for 10 min), and the supernatant was collected. The G6PDH activity in the supernatant was measured through the continuous reduction of NADP at 30°C in a spectrophotometer (λ = 340 nm) as described by Bergmeyer *(1)*. One G6PDH unit was defined as the amount of enzyme catalyzing the reduction of 1 μmol of NADP/min under the assay conditions. Each determination was made in triplicate and the SD was determined to be 2%. No significant decrease in G6PDH activity was detected after storing the supernatant for 5 h on ice at room temperature.

The G6PDH production was calculated through the equation:

$$P = (U/X_e) \times X \quad (1)$$

in which P = production of G6PDH (U/L_{medium}); U = G6PDH activity in the supernatant (U/mL); X_e = cell concentration in the suspension submitted to disruption (g_{cell}/mL), and X = cell concentration in the fermenting medium (g_{cell}/L_{medium}).

Calculation of Kinetic Parameters

The specific cell growth rate (μ_x), specific substrate consumption rate (μs) and specific G6PDHproduction rate (μ_P) were defined as follows:

$$\mu_x = (1/X) \times dX/dt \quad (2)$$

$$\mu_s = (1/X) \times dX/dt \quad (3)$$

$$\mu_p = (1/X) \times dX/dt \quad (4)$$

The derivatives dX/dt, dS/dt, and dP/dt were calculated according to the method proposed by Le Duy and Zajic *(7)*. The substrate-to-cell conversion factor ($Y_{X/S}$) and the substrate-to-G6PDH conversion factor ($Y_{G6PDH/S}$) were calculated as the inclination obtained between the variation in cell concentration (ΔX) or G6PDH production (ΔP) to the glucose consumed (ΔS), respectively. The maximum enzyme (Pr_{G6pdh}) and cell (Pr_x) productivity were calculated as $\Delta P/\Delta t$ and $\Delta X/\Delta t$, respectively, in which Δt = interval of cultivation time. The generation time (tg) was calculated as proposed by Borzani *(8)*.

Results and Discussion

The effect of agitation:aeration ratio, expressed as k_La, on the production of G6PDH was studied through a batch culture of *S. cerevisiae*, using an initial glucose concentration of 17 g/L.

It was apparent that the k_La affected the G6PDH activity produced by the yeast (Fig. 1). The highest G6PDH production, which was about 252.0 U/L_{medium}, occurred at a k_La of 60 h^{-1} after 12 h of fermentation. At this point, all of the glucose was consumed (Fig. 2), and the cell concentration was equal to 5.71 g/L (Fig. 3).

Nevertheless, at a k_La of 15 and 230 h^{-1}, the glucose concentration in the medium became negligible after 11 and 7 h, respectively, while at the intermediary k_La values (60 and 135 h^{-1}), it became negligible after 5 h (Fig. 2). Furthermore, the G6PDH production and cell concentration were markedly low for a k_La of 15 h^{-1} as compared with any of the other k_La values tested (Figs. 1 and 3). This result indicates that glucose consumption and G6PDH and cell production are coupled events and strongly dependent on the aeration/agitation of the medium. Since the best production parameters were observed for a k_La of 60 and 135 h^{-1} (Table 1), we can assume that under these agitation/aeration conditions the optimum dissolved O_2 concentration in the medium occurs.

Table 1
Generation Time (*tg*), Glucose-to-Cell Conversion Factor ($Y_{X/S}$), Glucose-to-G6PDH conversion Factor ($Y_{G6PDH/S}$), and Maximum Cell (Pr_x) and Enzyme (Pr_{G6pdh}) Productivities Related to Batch Fermentation of *S. cerevisiae* Under Different Initial k_La Values

	kLa (h^{-1})			
Parameter	15	60	135	230
tg (h)	4.3	1.8	1.8	2.5
$Y_{X/S}$ (g_{cell}/g_{glu})	0.14	0.21	0.21	0.19
$Y_{G6PDH/S}$ (U/g_{glu})	3.6	10.1	10.2	9.3
Pr_X (g_{cell}/[L·h])	0.24	0.69	0.69	0.49
Pr_{G6PDH} (U/[L·h])	10.6	31.8	30.3	23.3

The specific G6PDH production (μ_P) and the specific growth rate (μ_x) can be reasonably correlated by the approach proposed by Luedeking and Piret *(9)*, as shown in Fig. 4. The least square linear regression was $\mu_P = 32.4\mu_x + 0.013$ ($r^2 = 0.80$). Assuming that the linear coefficient is negligible, G6PDH formation and growth are associated events.

It must be mentioned that G6PDH formation did not vary significantly for k_La higher than 60 h^{-1}, a result likely related to the fact that this enzyme is influenced more by cytoplasm $NADP^+$:NADPH than adenosine dipho-sphate:adenosine triphosphate (ADP:ATP) ratio. The latter, is highly dependent on available oxygen in the medium, whereas the ratio of $NADP^+$/NADPH depends heavily on the reductive metabolism *(10)*. Furthermore, two other factors can contribute to the relative independence of G6PDH on the dissolved oxygen. First, there is the reversible conversion of G6P to 6-phosphoglucono-δ-lactone catalyzed by G6PDH *(10)*. Second, compared to hexokinase (the enzyme that forms G6P), G6PDH is far more sensitive to dissolved oxygen *(11)* and has a K_M fivefold lower *(12)*. This supports the fact that G6PDH can normally furnish the intermediate metabolites needed for the cell growth, even when hexokinase activity is depressed (e.g., high ATP:ADP ratio).

In the present case, we can assume that the k_La of 60 h^{-1} delivers an adequate amount of dissolved oxygen in the medium to force selectively the metabolic pathways to high growth and glucose conversion. This is clearly noted by the production parameters given in Table 1, which indicate at a k_La of 60 or 135 h^{-1} with the following values: $Y_{X/S}$ (0.21), $Y_{G6PDH/S}$ (10.1 and 10.2 U/g_{glu}), Pr_x (0.69 g_{cell}/[L·h]) and Pr_{G6PDH} (31.8 and 30.3 U/[L·h]). In addition, for those k_La values the generation time (*tg*) was the lowest and equal to 1.8 h (Table 1).

It must be realized that in all experiments, the cell growth continued even when all glucose was consumed (Fig. 3). This can easily be understood if we consider that after glucose is consumed another substance becomes a substrate to the yeast. In this case, the likely candidate is ethanol, which

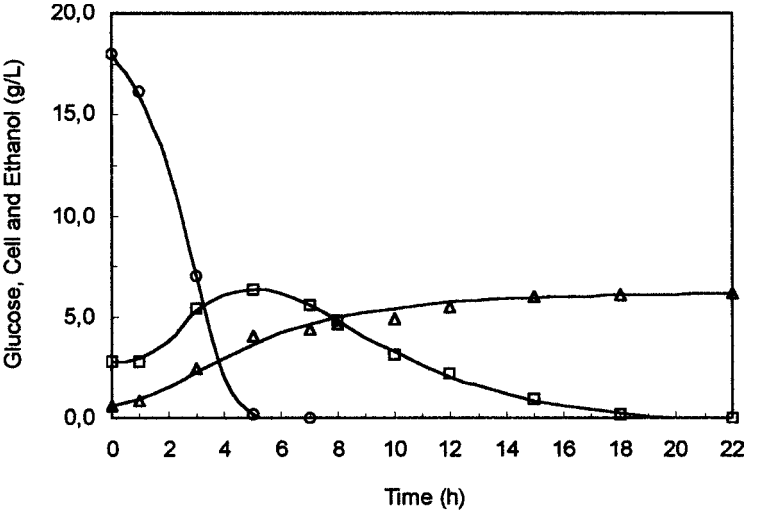

Fig. 5. Variation against time of glucose (s), cell (Δ), and ethanol (h) concentrations for *S. cerevisiae* grown at k_La of 60 h^{-1}.

accumulates as glucose that has been taken up by the yeast (Fig. 5). According to Rose and Harrison *(13)* and Rehm and Reed *(14)* the production of ethanol by *S. cerevisiae*, even under high aeration, is a characteristic of this specie of yeast. In other words, the Pasteur effect is not prevalent in *S. cerevisiae*.

As G6PDH is a constitutive and growth-related enzyme, a viable process for its production would be in a steady-state continuous culture, as has previously been suggested for other enzymes produced with the same characteristics, such as invertase *(15)*. In this case, according to Doin *(16)*, an estimation of the more suitable dilution rate (D) for the continuous process must be made from the data attained in batch culture. This can be accomplished by plotting the variation in growth rate (dX/dt) vs cell concentration (X) (Fig. 6). By setting 12 h of batch fermentation as the period in which the highest G6PDH production was attained (Fig. 1), the correspondent cell concentration was 5.71 g/L (k_La = 60 h^{-1}). According to Doin *(16)*, the angular coefficient of the line linking directly the points (0, 0) and (5.71, 0.10), as depicted in Fig. 6, would be a reasonable estimation of D, which in this case should be approx 0.02 h^{-1} (i.e., 0.10 g/[L·h] divided by 5.71 g/L). An alternative approach for G6PDH production would be by direct extraction from residual distillery yeast, which is an open opportunity for ethanol-producing countries (such as Brazil).

Conclusion

It can be concluded that the high level of G6PDH formation by *S. cerevisiae* occurred at k_La of 60 h^{-1} after 12 h of batch culture, and as G6PDH formation is related to cell growth, a perspective should be to produce the enzyme through a continuous culture at dilution rate of 0.02 h^{-1}.

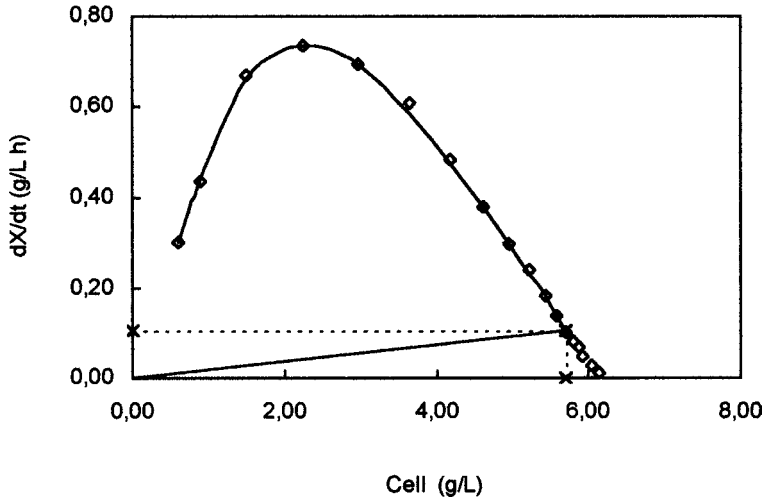

Fig. 6. Growth rate (dX/dt) vs cell concentration (X) for estimating dilution rate (D) for steady-state continuous culture based on data obtained from discontinuous culture carried out at initial k_La of 60 h^{-1}.

Acknowledgments

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